shRNA-mediated RNA interference as a tool for genetic synthetic lethality screening in mouse embryo fibroblasts

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Abstract Previously, we demonstrated the establishment of synthetic lethality screening in cultured somatic human cells, or mouse embryo fibroblasts (MEFs), for chemicals or mutant genes synergistically lethal with a mutated gene of interest. Here, we show in MEFs that the usage of RNA interference-based genetic suppressor elements encoding short hairpin RNAs (shRNAs) enables for genetic synthetic lethality screening at a frequency much higher than that achieved before with short truncated sense and antisense RNAs. These findings open up the possibility of using in mammalian cells genome-wide shRNA libraries for genetic synthetic lethality screening at the multigene level.

Keywords: Synthetic lethality screen; RNA interference; Genetic suppressor elements

1. Introduction

The completion of the comprehensive sequencing of the human genome [1,2], as well as the finishing of the draft sequence of the mouse genome [3] has created an urgent need to develop additional functional genomic techniques for the elucidation of gene function.

The genetic synthetic lethality screen in yeast is a very powerful functional genomic method because it can reveal not only interactions between gene products with direct physical contacts, but also interactions along the same or parallel pathways [4]. Previously, we established chemical and genetic synthetic lethality screens in cultured human cells [5,6]. More recently, we applied the chemical synthetic lethality technology also to mouse embryo fibroblasts (MEFs) [7]. Here, we sought to expand the genetic synthetic lethality screening to MEFs. Previously, we established this screening in human cells, based on the usage of dominant-negative genetic suppressor elements (GSEs) of either the expressible short sense or antisense RNA. The frequency of a bona fide GSE was only 1–2% from among the total truncated sense and antisense RNAs [6]. Such low frequency of GSEs among the truncated sense and antisense cDNA constructs was already observed by others [8]. Consequently, in order to ablate the activity of a single cellular gene among say 5000 genes expressed in a particular mammalian cell, one would have to generate and screen at least 250 000–500 000 mammalian cell clones, a formidable task by all means. However, the emergence of gene ablation technologies based upon the RNA interference (RNAi) phenomenon has opened up new experimental opportunities [9]. In particular, the generation of vectors directing the synthesis of short hairpin RNAs (shRNAs) that are processed to small interfering RNAs (siRNAs) enables for stable persistent suppression of endogenous gene expression [10,11]. The pSUPER mammalian expression vector was designed to direct the synthesis of shRNA transcripts via the DNA polymerase III promoter that could be processed to siRNAs, such that a 19-nt inverted repeat is separated by a 9-nt loop [12].

Here, we apply pSUPER plasmid vectors in a genetic synthetic lethality screen in MEFs. We identify, at high frequency, shRNAs capable of efficient suppression of a cellular gene, which is synthetic lethal with the gene of interest. Thus, we establish a genetic synthetic lethality screen in mammalian cells using stable gene suppression via RNAi.

2. Materials and methods

2.1. Plasmids construction

The construction of the episomal *HPRT1*-tpzGFP survival plasmid and the pIS integrating sphGFP vector with a zeo^R dominant selectable marker has been previously described [5,6]. pRSV-neo plasmid was constructed by replacing the CMV promoter within pCMV-neo vector with a RSV promoter from pREP4 vector (Invitrogen). The pSUPER vector generated in Agami's group was described [12]. To generate pSUPER-GMP synthetase (pSUPER-GMPS), three pairs of target sequence oligonucleotides were designed according to ''siRNA user guide'' (T. Tuschl, personal communication). Each one contains a unique 19-nt double stranded human GMPS target sequence, present as an inverted repeat, separated by a loop of 9-nt spacer, and transcribed from a DNA polymerase-III H1-RNA gene promoter [12]. The three pairs of DNA oligonucleotides have the following sequences:
PSUPER-GMPS1 F:
5′ gatccccCATTCTCAGGGAGTCTGGGttcaagagaCCCAGACTCCCTGAGAATGTttttggaaa 3′
pSUPER-GMPS1 R:
5′ agcttttccaaaaaCATTCTCAGGGAGTCTGGGtctcttgaaCCCAGACTCCCTGAGAATGggg 3′
pSUPER-GMPS2 F:
5′ gatccccGGAGCTGTTGTCATTCTGGttcaagagaCCAGAATGACAACAGCTCCtttttggaaa 3′

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Abbreviations: GMP, guanosine monophosphate; GMPS, GMP synthetase; HPRT1, hypoxanthine-guanine phosphoribosyl transferase 1; shRNA, short hairpin RNA; MEF, mouse embryo fibroblasts

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pSUPER-GMPS2 R:
5′ agcttttccaaaaaGGAGCTGTTGTCATTCTGGtctcttgaaCCAGAA-TGACAACAGCTCCggg 3′
pSUPER-GMPS3 F:
5′ gatccccCTTGAAACCAGAGGAGGTTttcaagagaAACCTCCTCT-GGTTTCAAGtttttggaaa 3′
pSUPER-GMPS3 R:
5′ agcttttccaaaaaCTTGAAACCAGAGGAGGTTtctcttgaaAACCTC-CTCTGGTTTCAAGggg 3′
pSUPER-GMPS1 contains a 19 nucleotide sequence corresponding to nucleotides 1791–1809 downstream from the transcription start site of the human GMPS cDNA, pSUPER-GMPS2 contains nucleotides 76–94, and pSUPER-GMPS3 contains nucleotides 1068–1086. pSUPER-GMPS1 and -GMPS2 also share 100% homology to the mouse GMPS gene, while pSUPER-GMPS3 has four mismatches with the corresponding mouse gene.

2.2. Cell culture, transfections and reagents

The HPRT1-deficient 2TGOR cells (a kind gift from H. Ozer, UMDNJ) are derivatives of the MEFs BALB/c3T3. SKBR3 cell line is an ERα-negative breast cancer cell line and was purchased from the ATCC. The 2TGOR and SKBR3 cell lines were maintained in DMEM, supplemented with 10% fetal calf serum (FCS) and 4 mM L-glutamine. All transfections were carried out using LipofectAMINE Plus reagent (Life Technologies). pSUPER vectors were cotransfected with pRSV-neo at a 10 to 1 molar ratio, and selection in G418 (Calbiochem) was carried out at 1200 μg/ml for 2TGOR cells and at 750 μg/ml for SKBR3 cells. Mycophenolic acid (MPA) was obtained from Eli Lily (Indianapolis, IN). Synthetic lethality screening experiments performed in the Zeo-5 cells were carried out in the presence of 12.5 μg/ml guanine.

For cell toxicity experiments, transfections were performed in 6-well microplates without hygromycin B selection, the cells were passaged to 10 mm plates two days later, and then G418 selection was carried out. The obtained colonies were fixed with methanol, stained with Giemsa reagent, and photographed.

Fluorescent scanning of microtiter plates was previously described [7].

2.3. Quantitative real-time RT-PCR analysis

Total RNA was isolated from a pool of transfectants of each pSUPER construct by EZ-RNA isolation kit (Biological Industries, Israel) and transcribed into cDNA using EZ-first strand cDNA synthesis kit (Biological Industries, Israel). Real-Time PCR was performed with FastStart DNA master SYBR Green I kit (Roche) at the LightCycler instrument (Roche), according to the manufacturer's instructions. The DNA primer sequences were designed for murine GMP synthetase as follows (sense 5′-GGTTTGCTGGGAAAATCAGC-3′ and antisense 5′-CAGGAATCTCATTGCCAGG-3′) and for murine β-actin as follows (sense 5′-CTGAGAGGGAAATCGTGCGT-3′ and antisense 5′-TGTTGGCATAGAGGTCTTTACGG-3′). The cycling conditions for the GMP synthetase cDNA included preincubation for 15 min at 95 °C and 50 cycles comprised of 15 s at 95 °C, 10 s at 59 °C, 20 s at 72 °C and 5 s at 81 °C. For β-actin, the cycle was identical except for primer annealing performed at 53 °C and the final step done at 85 °C. At the end of each reaction, melting curve analysis was performed. Each assay included a standard curve of four serial dilution points. All samples were read at least in triplicates, and values were normalized for baseline expression and for expression of β-actin mRNA.

3. Results and discussion

3.1. The model system, construction of pSUPER-GMPS vectors, and testing their activity in mammalian cells

As a model system for the establishment of genetic synthetic lethality screening, the purine biosynthesis pathway was chosen (Fig. 1), as outlined before [5]. Briefly, biosynthesis of the essential metabolite GMP is achieved by either the de novo pathway or, when needed, via the salvage pathway catalyzed by the enzyme HPRT1. In the HPRT1-deficient MEF cell line

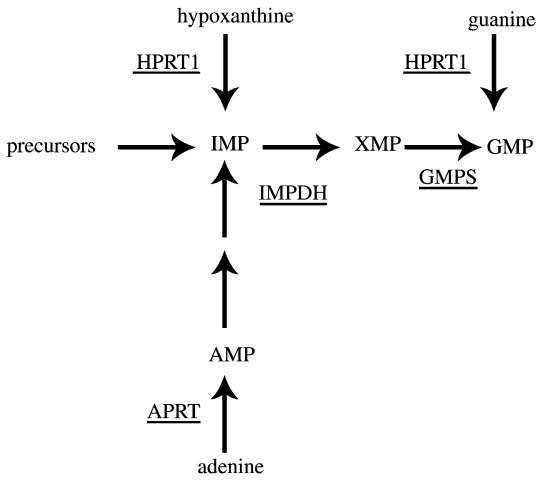

Fig. 1. De novo and salvage pathways of purine biosynthesis. Key enzymes are underlined. Arrows indicate the action of enzymes.

2TGOR, inhibiting the IMP dehydrogenase enzyme (IMPDH) or the GMP synthetase enzyme (GMPS), disrupts the de novo pathway, leading to synthetic lethality. Such selective pressures impose retention of the otherwise unstable EBV-based episomal survival plasmid expressing the salvage pathway's HPRT1 cDNA, serving as the gene of interest. Because the mouse, as well as the human, IMPDH enzyme activity is encoded by two different genes, an attempt was made to inhibit the single cellular GMPS gene rather than the two IMPDH genes. Accordingly, three pSUPER-GMPS vectors were designed and cloned as outlined in Section 2. These sequences were chosen to represent different regions of the GMPS transcript, and in order to allow versatility of usage in human and mouse cells, pSUPER-GMPS1 and pSUPER-GMPS2 share 100% homology to the mouse GMPS cDNA. The target human sequence present in pSUPER-GMPS3 has four mismatches with the corresponding mouse sequence.

We then turned to test the functionality of the pSUPER-GMPS constructs to suppress expression of the endogenous cellular GMPS gene. We assumed that stable blocking of GMPS expression in cells would ablate the de novo pathway of GMP synthesis, leading in HPRT1-deficient cells such as the mouse 2TGOR, to a synthetic lethal condition. For this purpose, equal numbers of 2TGOR cells were cotransfected by pRSV-neo and either the pSUPER empty vector, or each one of the three human GMPS sequence-containing pSUPER constructs, while selecting for colonies resistant to the G418 toxic chemical.

In comparison to empty pSUPER vector and pSUPER-GMPS3, the relative numbers of colonies residing in pSUPER-GMPS1 or pSUPER-GMPS2 transfected plates were severely reduced (Fig. 2A). Next, we tested while using the same approach the potential dominant-negative activity of these constructs also in human cells. For this purpose, we used the human breast cancer cell line SKBR3 but in the absence of the HPRT1 substrate guanine. As shown in Fig. 2B, the dominant-negative activity of the constructs is qualitatively similar with the pSUPER-GMPS1 and pSUPER-GMPS2 vectors being the active ones. Interestingly, pSUPER-GMPS3 whose target sequence is presumably identical to the SKBR3 one is totally inactive. Although we have shown that pSUPER-GMPS3 is transcriptionally active in human cells (data

not shown), failure in either its RNA processing to siRNA, or in its mRNA processing activity, could account for its lack of activity. Based on our limited experience, about 50% of the pSUPER constructs actually suppress the expression of the mRNAs against which they were designed for. Accordingly, in contrast to the 1–2% frequency of GSEs among truncated sense and antisense RNAs for human GMPS [6], two of the three shRNA constructs were found to impose effective RNAi in human as well as in mouse cells.

3.2. The pSUPER-GMPS mediated RNA interference suppresses cellular GMPS mRNA levels

We next examined directly whether expression of pSUPER-GMPS1 and -GMPS2 leads in 2TGOR-derived cells to RNAi mediated suppression of cellular GMPS mRNA. For this purpose we resorted to the Zeo-5 cell clone, which served as the model system for chemical synthetic lethality screening in MEFs [7]. This cell line is derived from the 2TGOR MEF and harbors on the one hand an integrated sphGFP transcription unit, the EBV-based episomal survival plasmid expressing the tpzGFP, and the human HPRT1 enzyme. The latter can complement the HPRT1-deficiency in the 2TGOR/Zeo-5 cells. Loss or retention of the episomal survival plasmid can be

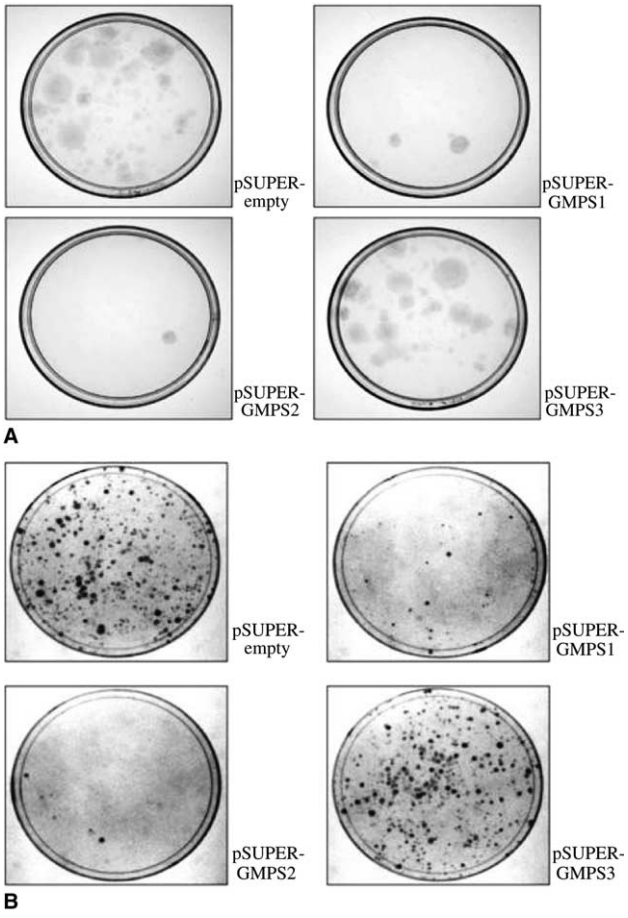

Fig. 2. pSUPER-GMPS conferred cell toxicity in the HPRT1-deficient 2TGOR MEF cell line (A) or in the SKBR3 human breast cancer cell line (B). Similar number of cells were stably cotransfected with pRSV-neo and either pSUPER empty or with each one of the three pSUPER-GMPS constructs. Two weeks after completing the selection for stable cell clones, the cells were fixed and stained for colonies visualization.

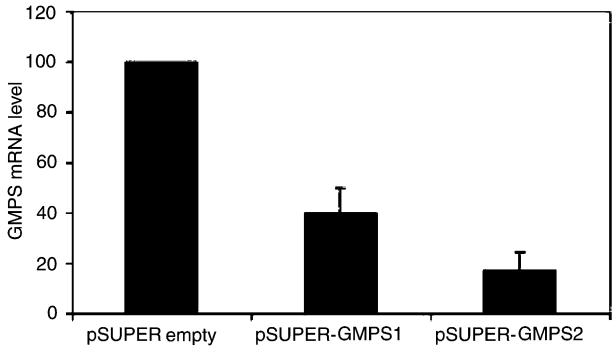

Fig. 3. RNA interference-mediated suppression of cellular GMPS mRNA. RNA was extracted from pools of transfectants and the mRNA level of GMPS was tested by real-time PCR. All samples were read at least in triplicates and values were normalized for β-actin mRNA expression. The data are represented as percent of the empty vector control value. The statistical significance was tested by t test, resulting in the value of probability of $P < 0.01$ for pSUPER-GMPS2 and $P < 0.05$ for pSUPER-GMPS1.

determined by measuring the fluorescence intensity ratio between tpzGFP and sphGFP readings. To generate a putative synthetic lethal condition, we cotransfected Zeo-5 cells, after removal of the hygromycin B selection pressure from the episome, with pRSV-neo and each one of pSUPER-GMPS1, -GMPS2, or the pSUPER empty vector. Stable clones were obtained by the selection for G418 resistance. We have then isolated about 20 clones from each transfection, while the remaining colonies in each set were pooled for total RNA extraction.

To compare the relative levels of the corresponding cellular GMP synthetase mRNA, we performed a quantitative real-time PCR analysis. In comparison to the endogenous GMP synthetase mRNA level of pSUPER empty vector-transfectants, the respective mRNA level of pSUPER-GMPS1 pooled transfectants drops by 60%, whereas in the better expressed pSUPER-GMPS2 vector (data not shown) Zeo-5 pooled transfectants there is an 83% decrease in mRNA levels (Fig. 3). As the levels of the cellular GMPS mRNA were normalized in each case against the murine beta actin mRNA levels, serving as an internal control (see Section 2), this shRNA mediated suppressing activity is obviously gene specific. Taking into account that total RNA was extracted from pooled MEF transfectants, rather than individual colonies (verified for maintenance of the target sequence transcription unit), GMPS mRNA decay levels may be considered as an underestimation for the potency of the respective pSUPER-GMPS constructs.

3.3. Expression of GMPS shRNAs confers synthetic lethality upon Zeo-5 cells

We next examined whether the expression of pSUPER-GMPS1 and -GMPS2 in Zeo-5 cells leads to retention of the episomal survival plasmid reflecting a synthetic lethal condition. As outlined above, we have isolated about 20 clones from transfection with pRSV-neo and each one of pSUPER-GMPS1, -GMPS2, or the pSUPER empty vector. The individual colonies were propagated and seeded into microtiter plates. Two weeks later, the microtiter plates were read for tpzGFP and sphGFP fluorescence. As shown in Fig. 4, in cells transfected by either pSUPER-GMPS1 or pSUPER-GMPS2 vector there is a shift in the fluorescence ratio distribution towards

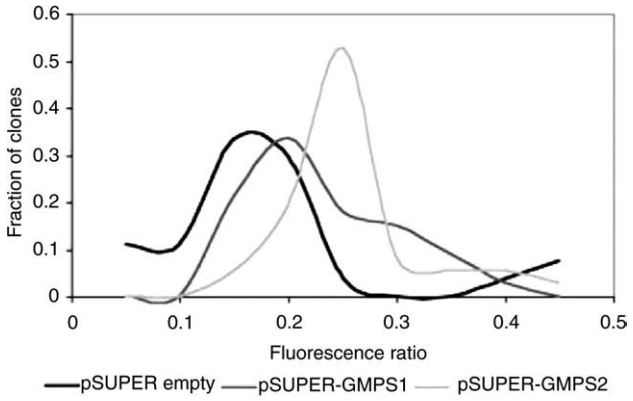

Fig. 4. Expression of GMPS sequence-containing shRNAs confers synthetic lethality. Zeo-5 cells were stably cotransfected with pRSV-neo DNA and either pSUPER-empty vector, pSUPER-GMPS1 or pSUPER-GMPS2. One month after the transfection, about 20 individual cell clones from each transfection were isolated and grown in microtiter plates in triplicates without the hygromycin B selection pressure. The tpzGFP to sphGFP fluorescence ratio was read two weeks after the cell seeding. Depicted is the fraction of cell clones (*Y*-axis) as a function of the GFPs fluorescence ratio (*X*-axis). The statistical significance was tested by *F*-test, resulting in the value of probability of 0.012 for pSUPER-GMPS2 and 0.025 for pSUPER-GMPS1.

higher normalized tpzGFP expression, as compared to MEF Zeo-5 cells transfected with pSUPER empty vector. High fluorescent ratio represents the retention of the GFP-tagged episomal survival plasmid. These data indicate that the previously observed cell death associated with either pSUPER-GMPS1 or pSUPER-GMPS2 induced RNAi (Fig. 2) reflects in each case the generation of a synthetic lethal condition (Fig. 4).

We have demonstrated in this work that the stable RNAi methodology can be incorporated into a genetic synthetic lethality screening technology. This has been demonstrated in MEF cells, thus opening up the application of the genetic synthetic lethality screening to the multitude of knockout mice, representing deficiencies in variety of potential genes of interest. Since the EBV-based episomal survival plasmid is practically the same in mouse and human cells [5,7], obviously the same methodology applies also to human cells. Importantly, several groups have reported recently the generation and usage of multi-gene human and mouse shRNA-encoding libraries [13,14]. Such libraries, constructed within retroviral vectors, can therefore be readily transduced into genetic synthetic lethality screening in human or MEF cell systems, grown in 96-well microplates, while monitoring periodically the tpzGFP-tagged episomal plasmid retention. In this respect, an alternative procedure for genetic synthetic lethality screening has been recently suggested by the makers of the shRNA expression libraries [13–15]. This one is based on the DNA bar-codes strategy enabling identification of individual shRNA construct in complex populations via microarray analysis. The availability of such human/mouse shRNA expression libraries coupled with our findings opens up the possibility of performing a relatively straightforward procedure for identification of genes synthetic lethal with a gene of interest; such as target genes for anticancer therapy linked via synthetic lethality relationship to mutated tumor suppressor genes.

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